# Resolution and Reconstitution of a Mammalian Membrane

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ABSTRACT The problem of the resolution and reconstitution of the inner mitochondrial membrane has been approached at three levels. (1) Starting with phosphorylating submitochondrial particles, a "resolution from without" can be achieved by stripping of surface components. The most extensive resolution was recently obtained with the aid of silicotungstate. Such particles require for oxidative phosphorylation the addition of several coupling factors as well as succinate dehydrogenase. (2) Starting with submitochondrial particles that have been degraded by trypsin and urea a resolution of the inner membrane proper containing an ATPase has been achieved. These experiments show that at least five components are required for the reconstitution of an oligomycin-sensitive ATPase: a particulate component, $F_1$, $Mg^{++}$, phospholipids, and $F_c$. Morphologically, the reconstituted ATPase preparations resemble submitochondrial particles. (3) Starting with intact mitochondria individual components of the oxidation chain have been separated from each other. The following components were required for the reconstitution of succinoxidase: succinate dehydrogenase, cytochrome *b*, cytochrome $c_1$, cytochrome *c*, cytochrome oxidase, phospholipids and $Q_{10}$. The reconstituted complex had properties similar to those of phosphorylating submitochondrial particles; i.e., the oxidation of succinate by molecular oxygen was highly sensitive to antimycin.

## INTRODUCTION

This talk will deal with two areas of research which have collided only recently. One is the field of membranology and the other is the field of oxidative phosphorylation. In spite of the fact that neither field was moving very fast, the impact of the collision was considerable. Investigators in these two areas of research have learned a great deal from each other, particularly since very different qualifications are required to be an expert in each field. The membranologist must know physical chemistry, electrophysiology, and electron microscopy. He must be a man of decision, so that he can choose among hundreds of electron micrographs. The expert in oxidative phosphorylation, on the other hand, should be a versatile biochemist with a great deal of patience, willing to get lost in a jungle of unknown phenomena. He must have a lot of imagination so that he can see intermediates, even if there are none.

There is a story about Bernard Shaw who was once asked by a beautiful lady to marry her, because their offsprings would be wonderful specimen of great beauty and brilliant minds. Shaw rejected the offer because he was worried that the offsprings may acquire his looks and her brains. Still, it seems that on the whole the union between membranology and oxidative phosphorylation has had an invigorating effect on the products. We can now see membrane physiologists using biochemical assays, e.g. measuring the hydrolysis of ATP; and we can see biochemists using the electron microscope in the study of oxidative phosphorylation.

## TWO HYPOTHESES OF OXIDATIVE PHOSPHORYLATION

Before discussing our work on the reconstitution of the inner membrane of mitochondria, I should like to recapitulate briefly the role of the membrane

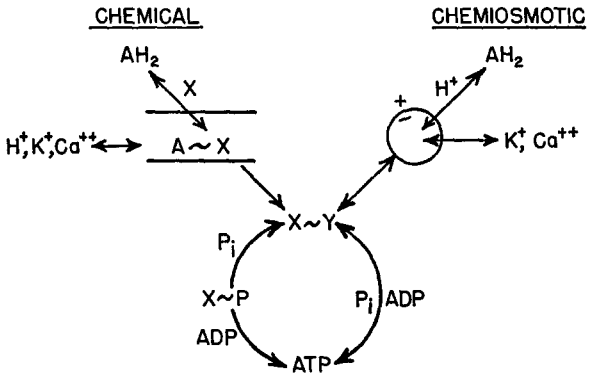


FIGURE 1 Similarities and differences in the two hypotheses of oxidative phosphorylation.

in oxidative phosphorylation. There are two major hypotheses of the mechanism of ATP production during substrate oxidation. According to the chemical hypothesis the membrane plays the role of an organizer which assembles the catalysts of oxidation and of phosphorylation to a functionally efficient multienzyme system. In the chemiosmotic hypothesis, as formulated by Mitchell (1), the membrane has an additional role acting as an insulator separating charges inside and outside of a vesicular structure. As shown in Fig. 1, the formulations of the two mechanisms have a number of features in common. According to the chemical hypothesis, the intermediate $X \sim Y$ is formed by transformation of a high-energy intermediate of the oxidation chain ($A \sim X$), whereas in the chemiosmotic hypothesis $X \sim Y$ is derived from the membrane potential and a pH gradient which are created during proton and electron flow through the "coupling membrane." Thus, in the Mitchell hypothesis, proton translocation is the primary process whereas in the chemical hypothesis, it is a secondary process. Jagendorf and Uribe (2) have shown

that a pH gradient can be used in chloroplasts to generate ATP from ADP and Pi . On the other hand, Cockrell, Harris, and Pressman (3) have shown that in mitochondria a potassium gradient can be used to generate ATP. It is apparent, therefore, that these ion-translocating systems are in equilibrium with a high-energy intermediate of oxidative phosphorylation and it is impossible at the present time to decide whether proton translocation is a primary or a secondary event in the pathway of oxidative phosphorylation. The arguments are quite eloquent on both sides and I have attempted to draw up a scoreboard to evaluate the evidence (Table I).

TABLE I

SCOREBOARD OF EXPERIMENTAL EVIDENCE FOR THE CHEMICAL AND CHEMIOSMOTIC HYPOTHESIS OF ENERGY GENERATION DURING OXIDATIVE PHOSPHORYLATION AND PHOTOPHOSPHORYLATION

| | Chemical | Chemiosmotic |
|---|---|---|
| Role of membrane | − | + |
| Action of uncoupling agents | − | + |
| Isolation of high-energy intermediates | − | ± |
| Ion transport | + | − |
| $^{32}P_i$-ATP exchange<br>ADP-ATP exchange<br>$H_2{}^{18}O$ exchanges | + | − |

## SCOREBOARD

The fact that oxidative phosphorylation has as yet not been observed in the absence of vesicular membranes favors the chemiosmotic hypothesis. The dual function of $F_1$ and of some other membrane components which I shall mention later is also more easily understood in terms of Mitchell's formulations. Recent studies on the effect of uncoupling agents on model membranes (4) support the proposition of Mitchell that certain uncouplers, e.g. dinitrophenol, increase the permeability of the membrane to protons. The notable lack of success in the isolation of intermediates carries some weight in favor of Mitchell, since he only failed to isolate one intermediate ($X \sim Y$), while the proponents of the chemical hypothesis have failed to isolate three. On the other hand, some studies on ion transport, e.g. the translocation of 7 moles of $K^+$ per mole of ATP (3), are difficult to reconcile with the current Mitchell model. The exchange reactions listed in Table I as observed in mitochondria and chloroplasts are more readily explained by the chemical hypothesis which includes an intermediate $X \sim P$. This point is discussed extensively elsewhere (5) and will not be elaborated here, except to say that inclusion of an $X \sim P$ in the Mitchell hypothesis would not change its basic concept, although it would somewhat even the score with regard to the failure

of isolating intermediates. Similarly, the proponents of the chemical hypothesis could gracefully accept Mitchell's formulation on the mode of action of uncouplers since ion translocation is reversibly linked to the high-energy intermediate (Fig. 1) and thus can give rise to an uncoupling mechanism by dissipation of energy.

It seems to me that the most decisive answer depends on the successful resolution and reconstitution of a system of oxidative phosphorylation. If such a reconstitution can be achieved without formation of vesicular membranes, ion translocation can be regarded as a secondary process. If vesicles

TABLE II

RECONSTITUTION OF SUCCINOXIDASE AND SUCCINATE-CYTOCHROME *c* REDUCTASE

| Reconstituted complex | Succinoxidase | Succinate-cytochrome *c* reductase |
|---|---|---|
| | *mμatoms oxygen/min/mg* | *mμmoles cyt. c reduced/min/mg* |
| Complete system | 170 | 830 |
| " " minux succinate dehydrogenase | 13 | 19 |
| " " minus cytochrome *b* | 12 | 39 |
| " " minus cytochrome $c_1$ | 12 | 25 |
| " " minus phospholipid | 84 | 39 |
| " " minus $Q_{10}$ | 25 | 52 |
| " " minus cytochrome oxidase | 7 | — |
| " " plus antimycin A | 0 | 6 |

Conditions of reconstitution are described in detail elsewhere (6). They were not optimal for maximal rates as discussed in the text.

are required the current formulations of the chemical hypothesis are clearly inadequate.

In the hope that a resolution and reconstitution of a functional mitochondrial membrane can be achieved, we have conducted experiments with the inner membrane of beef-heart mitochondria at three levels; (*a*) resolution and reconstitution of the oxidation chain; (*b*) resolution and reconstitution of the oligomycin-sensitive ATPase; and (*c*) resolution of coupling factors and reconstitution of oxidative phosphorylation.

### *Resolution and Reconstitution of Succinoxidase*

I shall review briefly the work on the reconstitution of succinoxidase carried out in collaboration with Dr. S. Yamashita (6) and I shall emphasize the major lessons learned from these studies. In Table II the dependency on the various components used for the reconstitution of succinate oxidase and of succinate-cytochrome *c* reductase is shown. Omission of succinate dehydrogenase, cytochrome *c*, cytochrome $c_1$, $Q_{10}$, or cytochrome oxidase from

the complete system resulted in loss of succinoxidase activity. The dependency on phospholipids was only partial. However, in view of the complete dependency of succinate-cytochrome *c* reductase on the addition of phospholipid, the incomplete dependency of the succinoxidase complex is likely due to the presence of residual phospholipids in the cytochrome oxidase preparation. The rates of these reconstituted systems are comparable with those obtained with submitochondrial particles capable of catalyzing oxidative phosphorylation. For convenience of assay, relatively short incubation times for reconstitution were chosen in these experiments. As will be shown

TABLE III
PROTECTION OF CYTOCHROME *b* BY SUCCINATE AND GLYCEROL

| Additions | Variant conditions during cleavage: Glycerol + succinate | Succinate | Glycerol |
|---|---|---|---|
| | *mμmoles cytochrome c reduced/min/mg* | | |
| Exp. 1 | | | |
| Cytochrome *b* (variant) | 98 | 9 | 8 |
| Cytochrome $c_1$ (obtained with glycerol and succinate) | | | |
| Exp. 2 | | | |
| Cytochrome $c_1$ (variant) | 98 | 130 | 152 |
| Cytochrome *b* (obtained with glycerol and succinate) | | | |

In these experiments, either cytochrome $c_1$ (Experiment 1) or cytochrome *b* (Experiment 2) were prepared in the presence of both succinate and glycerol and were added during the reconstitution of the succinate-cytochrome *c* reductase complex. Cytochrome *b* (Experiment 1) or cytochrome $c_1$ (Experiment 2) were prepared in the presence of the components listed in the columns on the right. Experimental conditions are described in detail elsewhere (6).

below, prolonged incubations gave reconstituted complexes with considerable higher rates of cytochrome *c* reduction sometimes exceeding those observed in submitochondrial particles.

The key observation that made these reconstitutions possible was the successful resolution of the *b*-$c_1$ complex with 1.33 M guanidine in the presence of succinate and 16% glycerol. Deletion of either succinate or glycerol resulted in loss of reconstitutive activity. In order to establish which membrane component was protected by these agents, reconstitution experiments were carried out with cytochrome *b* prepared in the presence of succinate and glycerol, while cytochrome $c_1$ was prepared with only one of the protecting agents being present, and vice versa. It can be seen from Table III that cytochrome *b* exposed to guanidine in the absence of either glycerol or succinate was virtually inactive whereas neither glycerol nor succinate was required for the preservation of cytochrome $c_1$.

A second interesting observation was made on the requirement for phospholipid. A few selected experiments with different phospholipids are shown in Table IV. It can be seen that crude mixtures as well as purified phospholipids were effective in aiding the reconstitution. Considerable lengths of time of incubation at 37°C were required for maximal activity. For example, with soybean phospholipids optimal values were obtained only after 4 hr. With mitochondrial phospholipids the final rates of succinate oxidation were actually somewhat lower, but the reconstitution process was more rapid. Cardiolipin was not a satisfactory phospholipid for reconstitution, but short incubation at 0°C yielded actually better reconstitution than incubation at 37°C, which seemed to give rise to secondary inactivation. Purified phos-

TABLE IV

EFFECT OF PHOSPHOLIPIDS AND INCUBATION CONDITIONS ON RECONSTITUTION OF SUCCINATE-CYTOCHROME *c* REDUCTASE

| Phospholipids added | Reconstitution conditions | |
|---|---|---|
| | 20 min at 0°C | 2 hr at 37°C |
| | *μmoles cyt. c reduced/min/mg* | |
| Soybean phospholipids | 0.11 | 0.83 |
| Mitochondrial phospholipids | 0.08 | 0.81 |
| Cardiolipin | 0.31 | 0.06 |
| Egg lecithin | 0 | 0.37 |
| Phosphadidyl ethanolamine | 0.14 | 0.86 |

Experimental conditions are described in detail elsewhere (6).

phatidyl ethanolamine was very suitable. Many other purified phospholipids were tested and considerable variations were noted but since it is difficult to evaluate at the present time the contributions due to chemical structure and due to the state of physical aggregation, I shall abstain from a more detailed discussion.

In contrast to the other components, the preparation of cytochrome *b* was not soluble and we have thus far not been able to solubilize the preparation without damaging its reconstitutive capacity. Soluble preparations of cytochrome *b* described in the literature were found to be inactive in reconstitution experiments. Finally, it should be noted that succinate dehydrogenase was the only nonheme iron protein required for the reconstitution of an antimycin-sensitive succinoxidase.

*Resolution and Reconstitution of Rutamycin-Sensitive ATPase*

Extraction of mitochondrial particles with sodium cholate and fractionation with ammonium sulfate has yielded a fraction ($CF_o$) which was free of cytochrome oxidase and contained only traces of other oxidative enzymes and

phospholipids (7). On addition of mitochondrial ATPase ($F_1$) to $CF_o$ the hydrolytic activity was suppressed, but could be restored on addition of phospholipids (Table V). In contrast to soluble ATPase this reconstituted, particulate ATPase was sensitive to oligomycin or rutamycin. Electron microscopy revealed that the reconstituted ATPase consisted of vesicular membranes that looked surprisingly like phosphorylating submitochondrial particles. These experiments have been published in detail (8) and will not be discussed further, but they raise the interesting question of the exact localization of oxidative enzymes in relation to the inner mitochondrial membrane. With the aid of specific antibodies and of hydrolytic enzymes, we have embarked recently (9) on a systematic exploration of the localization of various membrane components. A comparison of the susceptibility of mito-

TABLE V
RECONSTITUTION OF OLIGOMYCIN-SENSITIVE ATPase

| Additions | Without rutamycin | With rutamycin |
|---|---|---|
| | *μmoles $P_i$/10 min* | |
| $F_1$ | 1.2 | 1.2 |
| $F_1$ + $CF_0$ | 0.17 | 0.15 |
| $F_1$ + $CF_0$ + phospholipid | 1.03 | 0.3 |

Experimental conditions are described in detail elsewhere (7).

chondria and submitochondrial particles to antibodies against $F_1$, cytochrome *c*, and cytochrome oxidase clearly established that the inner membrane is highly asymmetric and contains constituents which can be reached by the antibodies (or by enzymes) only from one side of the membrane and not from the other.

Further resolution of $CF_o$ proved difficult, but it was observed that sonication of submitochondrial particles at a very alkaline pH yielded particles which did not confer rutamycin sensitivity to added $F_1$ unless another soluble factor was added. In earlier experiments crude preparations of $F_4$ served as a source of this factor (10), more recently purified preparations were used (11). We have referred to this factor as $F_c$ or conferral factor, while MacLennan and Tzagoloff (12) call it OSCP (oligomycin sensitivity conferral protein). The system is still very complex as illustrated by the reconstitution experiments shown in Table VI. It is apparent that at least two heat-labile components are required to confer rutamycin sensitivity to $F_1$. Exposure of both the soluble factor ($F_c$) or the particulate component to either 55°C or to trypsin (not shown in table) resulted in a loss of rutamycin sensitivity of the reconstituted ATPase. In contrast to the separated components, the

reconstituted complex was not inactivated by exposure to 55°C, thus providing another example for the allotopy phenomenon (13).

The exact role of $F_c$ and of the particulate component in the conferral of rutamycin sensitivity remains to be elucidated. It appears quite likely, however, that inhibitors such as rutamycin or dicyclohexylcarbodiimide do not

TABLE VI
MULTICOMPONENT SYSTEM OF RUTAMYCIN-SENSITIVE ATPase

| Additions | ATPase activity Without rutamycin | With rutamycin | Inhibition |
|---|---|---|---|
| | *μmoles $P_i$/10 min* | | % |
| TUA-particles + $F_1$ | 1.52 | 1.14 | 25 |
| TUA-particles + $F_1$ + $F_c$ | 1.40 | 0.20 | 86 |
| TUA-particles + $F_1$ + $F_c$ (heated) | 1.38 | 1.02 | 26 |
| TUA-particles (heated) + $F_1$ + $F_c$ | 1.73 | 1.57 | 9 |

Preparation of TUA-particles and $F_c$ are described in detail elsewhere (11). The heat inactivation of $F_c$ and TUA-particles was carried out at 50°C for 15 min and at 55°C for 12 min, respectively.

TABLE VII
MASKING OF PARTICULATE ATPase BY ADDITION OF $F_c$ AND UNMASKING BY PHOSPHOLIPIDS

| Additions | ATPase activity Without rutamycin | With rutamycin | Inhibition |
|---|---|---|---|
| | *μmoles $P_i$/10 min* | | % |
| TUA-$CF_0$ + $F_1$ | 1.03 | 1.07 | 0 |
| " + $F_1$ + $F_c$ | 0.19 | 0.15 | 11 |
| " + $F_1$ + $F_c$ + P-lipids | 0.77 | 0.13 | 83 |

The preparation of phospholipid-depleted TUA-$CF_0$ and the experimental conditions of reconstitution are described in detail elsewhere (14).

act on $F_1$ itself but indirectly; e.g., by preventing the activation of the ATPase by phospholipids. This possibility was suggested by experiments with phospholipid-depleted particles (TUA-$CF_0$). These particles have been shown (14) to adsorb $F_1$ without conferring to it rutamycin sensitivity. As shown in Table VII, addition of $F_c$ to such a rutamycin-insensitive, particulate preparation resulted in a pronounced inhibition of ATPase activity, which was restored by phospholipids. It is thus conceivable that inhibitors such as rutamycin act by preventing the stimulation by phospholipids. It is apparent, moreover, that a stimulation by phospholipids, observed also with other membrane-bound ATPase systems, need not imply a requirement for a lipid at the active site, but may be due to an inhibition by another membrane component and reversal of inhibition by phospholipids.

Another point which may be of interest to membranologists is our earlier failure to discover a $Mg^{++}$ requirement for the binding of $F_1$ to the inner mitochondrial membrane. We found only recently (11) that monovalent cations substituted for $Mg^{++}$ and were effective even in the presence of ethylendiaminetetraacetate. Although much higher concentrations of monovalent than divalent cations were needed, the conventional buffer system contained sufficient monovalent cations to obscure the requirement for $Mg^{++}$ ions.

Since it is easy to get confused by the differences in the reconstitutions of oligomycin-sensitive ATPase from particles that are either deficient in $F_c$

TABLE VIII

RECONSTITUTION OF OLIGOMYCIN-SENSITIVE ATPase

| | | | | |
|---|---|---|---|---|
| 1. TUA-particles (deficient in $F_c$) | $\xrightarrow[Mg^{++}(K^+)]{F_1}$ | Active particulate ATPase | $\xrightarrow{F_c(OSCP)}$ | Particulate oligomycin-sensitive ATPase |
| 2. TUA-$CF_0$ (deficient in $F_c$ and in phospholipids) | $\xrightarrow{F_1}$ Active particulate ATPase (not sensitive to oligomycin) | $\xrightarrow{F_c}$ Inactive particulate ATPase | $\xrightarrow{\text{Phospholipids}}$ | Particulate oligomycin-sensitive ATPase |

or deficient in both $F_c$ and phospholipids these experiments are summarized in Table VIII.

*Resolution and Reconstitution of Oxidative Phosphorylation*

The third approach to the resolution of the inner membrane is the most gentle one. In contrast to the two previous methods which involve solubilization of the membrane by cholate or similar detergents, we have been trying to fractionate submitochondrial particles by "resolution from without"; i.e., by removing surface components by more gentle chemical or physical treatments.

In this category, the most resolved particles we have described thus far are the ASU-particles. They are submitochondrial particles that have been passed through a Sephadex column and then treated with urea. By addition of coupling factors they can be reconstituted (15) so that they are both morphologically and functionally indistinguishable from actively phosphorylating submitochondrial particles. In fact, the reconstituted particles have a P:O ratio which is considerably higher than that of the sonicated submitochondrial particles before resolution. With DPNH as substrate, P:O ratios approaching 3.0 are observed quite frequently.

We have recently succeeded in making even more highly resolved submitochondrial particles by an exceedingly simple procedure. While ex-

ploring different methods of negative staining for electron microscopy, we noticed that submitochondrial particles that were exposed to 2 % solutions of silicotungstate were depleted of the inner membrane spheres which we have identified as $F_1$ (8, 15). This observation led to a systematic study (16)

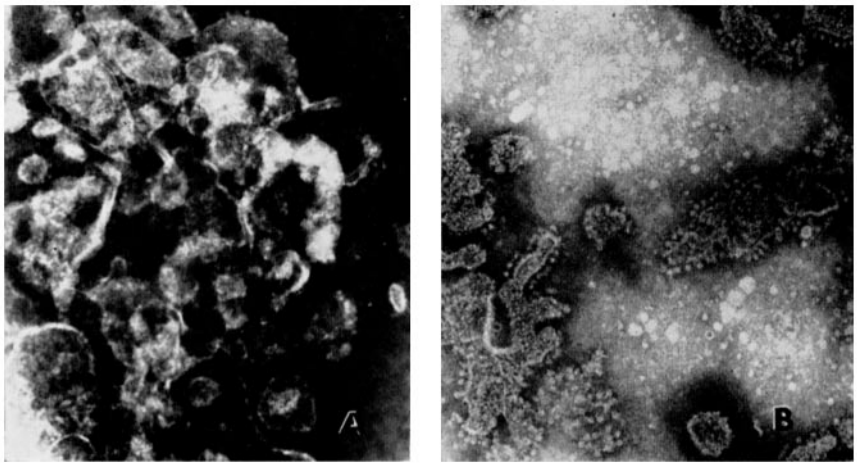

FIGURE 2 Electron micrographs of submitochondrial particles. (A) Submitochondrial particles after treatment with silicotungstate. (B) Silicotungstate-treated particles reconstituted with $F_1$. Negative stain with phosphotungstate. Final magnification was 80,000.

TABLE IX

STIMULATION OF OXIDATIVE PHOSPHORYLATION, $^{32}P_i$-ATP EXCHANGE, AND ENERGY-LINKED REDUCTION OF DPN IN STA-PARTICLES BY PREPARATIONS OF SUCCINATE DEHYDROGENASE

| Additions | P:O ratio | | *mμmoles $^{32}$ATP/ min/mg* | *mμmoles DPNH/ min/mg* |
|---|---|---|---|---|
| | DPNH | Ascorbate-PMS | | |
| STA-particles + coupling factors | 0.2 | 0.06 | 7 | 6 |
| " " + succinate dehydrogenase | 0.8 | 0.3 | 55 | 43 |

of the biochemical properties of particles treated with silicotungstate (STA-particles). In Fig. 2 the morphological reconstitution of STA-particles with $F_1$ is shown. Functional reconstitution of STA-particles proved more difficult until it was found that the particles were resolved with respect to succinate dehydrogenase. Most interesting was the finding that succinate dehydrogenase was required for reconstitution not only with succinate as substrate but also for phosphorylation associated with the oxidation of DPNH or ascorbate (Table IX). Furthermore, the $^{32}P_i$-ATP exchange rate and the ATP-de-

pendent reduction of DPN by succinate were dependent on addition of the succinate dehydrogenase preparation (Table IX). Although the presence of a contaminating new coupling factor in the highly purified succinate de-

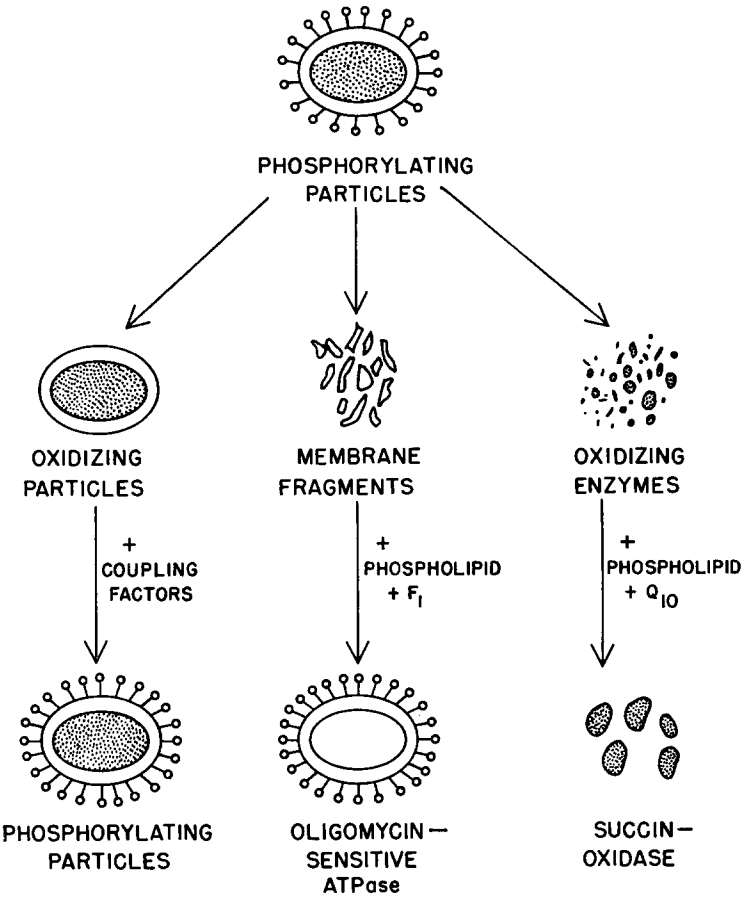


FIGURE 3 Three levels of resolution and reconstitution of submitochondrial particles. Left. Resolution from without; reconstitution with coupling factors. Center: Resolution of membrane components proper; reconstitution of oligomycin-sensitive ATPase. Right: Resolution of individual respiratory catalysts; reconstitution of succinoxidase.

hydrogenase preparation has not been ruled out, it is equally possible that this enzyme plays a structural as well as a catalytic role in the reconstitution of the inner mitochondrial membrane. This would represent yet another example for the dual role of membrane components (13).

In concluding, I should like to illustrate in Fig. 3 how we approached the problem of the resolution and reconstitution of the inner mitochondrial membrane at three levels. We hope that one day we shall be able to re-

constitute oxidative phosphorylation by supplying the necessary coupling factors to the oxidative chain. We believe that the studies of the reconstitution of the oligomycin-sensitive ATPase, the characterization of the coupling factors, and the reconstitution of the oxidation chain will help us in the design of such experiments.

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